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Thermoinactivation of Viruses: Report V. Thermoinactivation and thermostabilizing agents as a factor determining the level of VEE virus production in cell cultures.

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The general productivity of cells in mediums of different compositions was studied on the model of Venezuelan equine encephalomyelitis virus (VEE) and trypsinized chick embryo cells. Medium 199 with 2% bovine serum provided long-term and active virus production, the total yield of virus particles per one cell exceeding 10^4 PFU. In minimal essential medium (Earle's solution with 0,22% sodium bicarbonate) the total virus yield was more than 10 times lower despite the virus amplification to comparatively high titers. The most effective agents providing for adequate thermostabilization of VEE virus at 37 degrees (C) were found to be 2% bovine serum, 0,1% lactalbumin hydrolysate and 0,02% crystalline bovine albumin.

Thermoinactivation of virus particles at a temperature of cultivation is one of the factors influencing the level of virus production in cell cultures. We did research using the virus of equine Venezuelan encephalomyelitis (VEE) and trypsinized chick embryo cells as our models, on the combined influence on the virus harvest of therminactivation and the composition of storage mediums which to a significant degree determine the character of the duration and intensity of the process of heat loss of the infectious activity of the virus. The work was also an attempt at summarizing the research accumulated in the process of the thermoinactivation of viruses, on the thermostabilization and stimulating action of various additives to the feeding medium, in which basic attention was devoted to the high-molecular biological active substances.

Materials and Methods

The VEE virus and trypsinized chick embryo cells were used as described above (1, 4). Heating the virus was done according to the usual methods (7). The infectious activity of the virus was determined by the method of platelets under agar (5). Addition of lactalbumin hydrolysate was done from a calculation of the dry weight. The bovine serum before use underwent heating for 30 minutes at 56 degrees. Magnesium sulfate was used in the form of an official preparation (25%) likewise calcium chloride (10%).

Sterilization of 1% solution of carboxymeticellulose was produced with autoclavering, and 1% crystalline bovine alvumin with filtration through a millipore filter.

Results. Determining the general production of the VEE virus in optimal and minimal medium. The determined level of virus production, determination being done by titration by platelets, only in the period of exponential growth in activity corresponds to its general production. Further, as a result of the fact that the existing part of the virus population becomes inactive under the influence of incubation temperatures, determination of the general production demands application of special methods of research. It was established earlier that for production of the VEE virus in TKE cells, medium no. 199 with 2% bovine serum (3) is optimal. The minimal medium, which can still insure production of a sufficiently high harvest of VEE virus, is Earle's solution with 0,22% of sodium bicarbonate.

In these accumulation mediums with an infection multiplication of 5PFU/cell and a cultivation temperature of 37 degrees, determination was made of the speed of production and of the general harvest of the VEE virus in TKE cells according to the Purifoy et. al. method (12). In sketch 1 is shown a calibration diagram of the inactivation of the VEE virus at 37 degrees in medium No. 199 with 2% bovine serum and in Earle's solution with 22% sodium bicarbonate, determined as a result of repeated titration. In a series of parallel experiments we received, with titration by platelets under agar, curves of production of the infectious VEE virus. in TKE cells. The production speed is calculated by the formula:

$R(t) = 2,3\ G(t)\ [g'(t) - a]$, where $R(t)$ is the production speed of the virus in a given moment of time (t); $G(t)$ is the activity of the virus (in PFU/ml), defined on the graph of production of platelet forming activity of the virus; a is the fall in virus activity (in lg PFU/ml) for a unit of time determined on the graph of thermoinactivation of virus infection.

On the basis of determination of the production speed of the VEE virus VEE [R(t)] in various moments of time, the general production of the virus was calculated. Curves of the changes in the production speed of the virus and the general production of the virus in medium No. 199 with 2% bovine serum and in Earle's solution with 0,22% sodium bicarbonate are shown in sketch 2, a, b.

In comparison with the reproduction cycle in medium No. 199 with 2% bovine serum, in the Earle's medium with salt, the growth of the production speed of the virus slowed, and after 24 hours the production speed fell quickly. In the optimal medium of accumulation, the virus production lasted sufficiently long, with a comparatively high speed. As a result of this, the general harvest of virus in medium No. 199 with 2% bovine serum (10,3 lg PFU/ml) was significantly higher than in the minimal salt solution (9,1 lg PFU/ml).

A visual representation of the duration of the effective production of VEE virus in medium No. 199 with 2% bovine serum or 0,1% of hydrolysate lactalbumin gives the results of determination of the infection activity of the virus in cultures with change (full) every 24 hours of the culture liquid after a three-fold careful washing of the cells. The results shown in table 1, bear witness that on the second and third 24 hour period after infection, the speed of virus inactivation becomes equal to the speed of production of active virus particles, or exceeds it. Some exceeding in the separate samples, of the experiment figure in comparison with the control, was caused apparently by removal of the cell detritus, of the interferon, of the acidic and toxic products , or by the stimulating influence of the medium change on the cell metabolism.

Influence of thermostabilizing additives on production of the VEE virus. In sketch 3 is shown the inactivation of the VEE virus at 37 degrees in mediums of various composition. The most unstable virus is in Earle's solution with 0,22% of sodium bicarbonate; the most stable is in medium No. 199 with 2% of bovine serum or 0,1% of hydrolysate lactalbumin. The productivity of cell cultures, determined by harvesting the virus in 24 hours after infection, changed in dependence on the composition of the medium of accumulation and proportionally to the thermostabilizing properties of these mediums (table 2).

High-molecular additives exerted various influences on the stability of the VEE virus. In sketch 4 is shown virus inactivation in a medium No. 199 with 2% bovine serum and in Earle's solution with 0,22% $NaHCO_3$ in the presence of 0,02% crystalline bovine albumin and 0,1% carboxymetilcellulose. This last did not heighten the virus stability much, during the time that the bovine albumin stabilized the infection activity of the virus almost to the level caused by the presence of bovine serum. A 0,02% concentration of bovine albumin turned out to be most effective (sketch 5,a) for insuring a high production of virus in cell cultures; a further heightening ofthe content, the same as increasing the content of bovine serum to more than 2 - 5% (sketch 5 b), led to a steady decrease in virus output. The optimal concentration of carboxymetilcellulose during which the virus harvest increased by 0,2 - 0,3 lg, was 0,1%.

For finding the influence of bovine serum on the thermostability of the virus produced in medium No. 199 without serum, we determined the level of inactivation of the VEE virus at various temperatures. For incubation at studied temperature levels we took a virus grown in medium No. 199 without bovine serum, to which we added 2% bovine serum immediately before beginning to heat it. As a control, we used a virus grown in TKE cells in medium No. 199 with 2% bovine serum. The results of the experiments are shown in table 3. Both the populations of virus particles at the studied temperatures became inactive with identical speed.

The thermostabilizing influence of salt additives. At 37 degrees we studied the inactivation of the VEE virus in medium No. 199, containing bovine serum, and with the addition of a corresponding 12,5 and 5% of magnesium sulfate and calcium chloride. If 10% $MgCl_2$ hastened virus inactivation (sketch 6), then the addition to virus-containing medium No. 199 without serum of calcium chloride or magnesium sulfate heightened the virus stability to the level which was determined in the presence of 2% bovine serum. The simultaneous addition of bovine serum and salts of two-valence kations led to a summation of the stabilizing effect.

Taking into account this circumstance, the attempt was made to receive a higher virus VEE harvest by adding to the accumulation medium various concentrations of magnesium sulfate. However, beginning with a 1 - 2% concentration, the titers of the virus in the accumulation medium decreased in comparison with the control, proportionally to the concentration of magnesium sulfate which can be explained in all its forms by the toxic action of salt on the cells.

Discussion. Although TKE cells in minimal maintainance mediums can produce a sufficiently high virus titer (VEE), a detailed study of the production speed and the general virus harvest shows that virus-producing capabilities of the cells in these conditions is decreased. On the other hand, in mediums which are rich in nourishment substances, the virus production in one cell exceeds 10,000 PFU. As a result of the thermo-inactivation in virus-containing liquids, there accumulates, besides infectious virus, a significant quantity of inactivated, non-infectious virus particles. The difference in the durations of the period of virus production explains the fact that the difference in the maximal virus titers in medium No. 199 with 2% bovine serum and in Earle's solution with 22% $NaHCO_3$ (lg_{max} PFU/ml No. 199+2% bovine serum - lg_{max} PFU/ml Earle's + 0,22% soda = 0,65) is much less than the difference in production of PFU in a cell in these culture mediums (lg PFU/cell No. 199 + 2% bovine

serum - lg PFU/cell Earle's + 0,22% soda=1,2). Virus production calculated on one cell in the order of 10^4 PFU was noted in a series of cases for several arboviruses - the Sindbis viruses (11) and the Semlik forest virus (13). In this way in principle, a collection is possible of high virus yields during creation of optimal conditions of cultivation.

One of the factors insuring a high virus hield is the presence in the culture medium of thermostabilizing additives (see sketch 3, table 2). However the action of these substances is manysided. The presence of bovine serum does not exert influence on the production of more or less thermostable viruses. Its stabilizing action appears later - in virus - containing suspensions. From the other side, the removal of bovine serum leads to some decrease in the activity of the received virus-containing suspensions, probably as a result of the action of thermostable inhibitors contained in it (2). As a whole, the optimal contents in the a ccumulation medium of bovine serum is somewhat lower than the most favorable content for growth and life maintainance in TKE cells (1).

In other equal conditions the best action is exerted by substances having to some degree or other an expressed biological affinity to the surface structure of cells and viruses. This is well demonstrated with a comparison of the influence of bovine albumin and carboxymetilcellulose, the favorable activity of which is well known - activity, on the cell surface in non-serum mediums (8, 9). Yielding in effectiveness of activity to crystalline bovine albumin and in still greater degree to bovine serum and not exerting practical stabilizing influence on the thermoinactivation of viruses, carboxymetilcellulose somewhat increases the virus production as compared with the control, favorably acting on the cells.

The stimulating action of high-molecular additives was realized in several ways - because of heightening of the cell productivity, the increase of the period of active virus production, and as a result of their defensive activity on the surface of the cells and of the free viruses.

Absence of increased output of the viruses during addition of magnesium sulfate is explained apparently, by reasons of a twofold nature. The known thermstabilizing influence of cations on a series of viruses (10, 14), including the VEE virus (6), concerns the protein type of inactivation (3), which at 37 degrees does not always appear, and as a rule does not appear earlier than 48 - 72 hours (see sketch 6). In addition, concentrations of magnesium sulfate are effective for the thermosta bilization of virions, and exert a definitely toxic influence on TKE cells.

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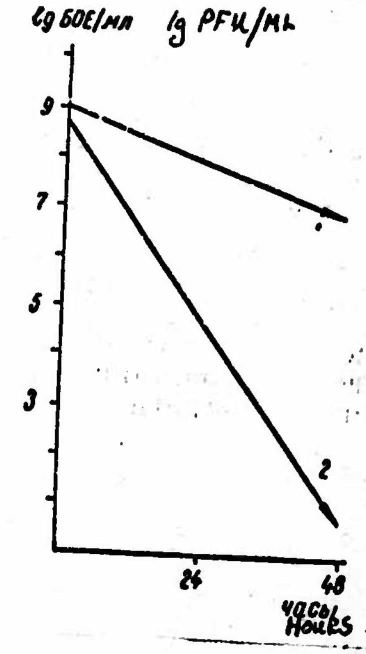
Sketch 1. Calibrated graph of the dynamic of thermo-inactivity of the VEE virus at 37 degrees in No. 199 medium with 2% bovine serum (1) and in Earle's solution with 0,22% sodium bicarbonate (2).



Sketch 2. Infectious activity (1), production speed (2) and general production of the VEE virus (3) in a TKE culture in No. 199 medium with 2% bovine serum (a) and in Earle's solution with 0,22% sodium bicarbonate (b).

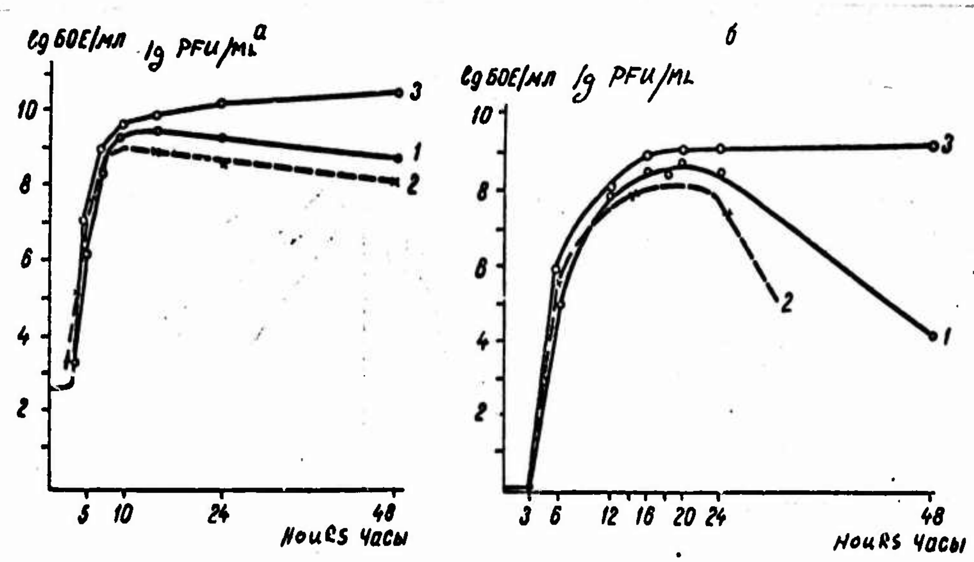


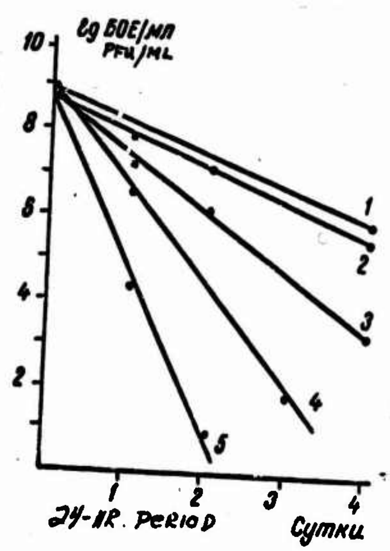
Table 1. Accumulation of the VEE virus in conditions of change of the medium every 24 hours (in lg PFU/ml).

Experiment conditions	24 hours	48 hours	72 hours
No. 199 medium with 2% bovine serum with a change of medium in 24 hours	8,7	8,3	5,7
The same without a medium change	8,6	7,5	5,0
No. 199 medium with 0,1% hydrolysate lactalbumin with a medium change in 24 hours	8,7	7,0	-
The same without a medium change	8,3	7,0	-

Sketch 3. Inactivation of the VEE virus at 37 degrees in No. 199 medium with 2% bovine serum (1), 1% hydrolysate lactalbumin (2), 1 % genatin (3), in medium No. 199 without additives (4), in Earle's solution with 0,22% sodium bicarbonate (5).

Sketch 4. Inactivation of VEE virus at 37 degrees in No. 199 medium with 0,02% bovine albumin (1) or 0,1% carboxymetilcellulose (2) and in Earle's solution with 0,22% sodium bicarbonate with 0,02% bovine albumin (3) or 0,1% carboxymetilcellulose (4).

3.



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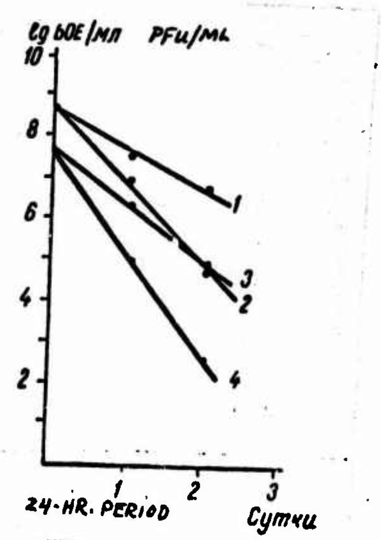


Table 2. VEE virus harvest in mediums of various compositions (statistics are the average results of 5 parallel experiments)

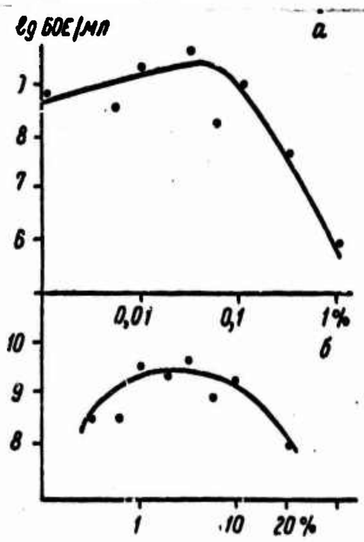
Composition of the growth medium	Earle's solution w.0,22% sodium bicarbonate	No. 199 medium	No. 199 medium & 1% gelatin	No. 199 medium w. 0,1% hydrolysate lactalbumin	No. 199 medium w. 2% bovine serum
VEE virus production in 24 hours (in lg PFU/ml)	8,5	9,0	8,9	9,2	9,3

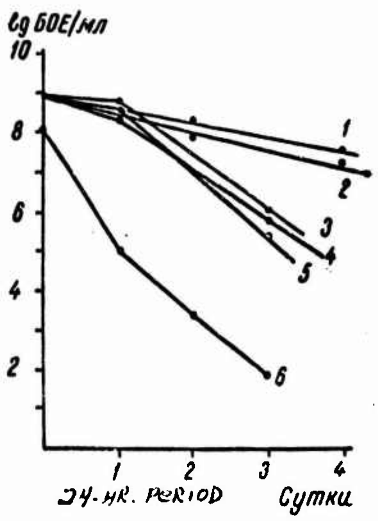
Table 3. Inactivation in No. 199 medium with 2% bovine serum of the VEE virus, grown in the presence of bovine serum and without it.

Heating temperature in degrees	Heating time (in minutes)	Fall in virus activity, grown in presence of bovine serum (in lg PFU/ml)	Fall in the activity of virus grown without bovine serum (in lg PFU/ml)
50	30	0,8	1,0
	60	1,7	2,0
52	40	2,5	2,6
55	15	2,3	2,0
	30	4,7	4,9

Sketch 5. Maximal activity of the VEE virus in a culture medium No. 199 depending on the content of albumin (a) or bovine serum (b)

5.





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Sketch 6. Inactivation of the VEE virus in No. 199 medium with various additives: 2% bovine serum and 12,5% sulfate of manganese (1), 2% bovine serum and 5% calcium chloride (2), 5% calcium chloride (3), 12,5% sulfate of magnesium (4), 2% bovine serum (5), 2% bovine serum and 10% manganese chloride (6).